VERONICELLA CUBENSIS AND *LAEVICAULIS ALTE*, INVASIVE SLUGS IN THE HAWIIAN ISLANDS: LIFE HISTORIES AND THE GUT MICROBIOME

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Abstract

Veronicella cubensis and *Laevicaulis alte* are widespread invasive slugs. Although they are voracious pests and known carriers of *Angiostrongylus cantonensis*, little is understood of the characters that make them successful invaders. To gain a more comprehensive understanding of these species, a study of their life histories was conducted. Slugs were reared in a lab setting to gather data on lifespan reproductive trends. The effect of temperature on juvenile growth was also determined by tracking the weight gain of slugs maintained in either a hot or cool temperature environment over the first six months of life. Observations of the egg laying behavior of *V. cubensis* prompted analysis of the gut microbiome and the possibility of transmission of the microbiome from parent to offspring from a substance laid on its egg masses. *Veronicella cubensis* reaches reproductive maturity at around 6 months of age and exhibits long duration mating and egg laying. The egg masses of both species contain a variable number of eggs, which hatch with a high success rate. Warmer temperatures cause faster weight gain in *V. cubensis* juveniles but not in those of *L. alte*. Microbiome analyses suggest the substance laid on *V. cubensis* egg masses comes from the adult slug hindgut, and the bacterial community on the surface of the eggs constitutes a component of the bacteria acquired by juveniles. These results are a valuable addition to the limited knowledge of the reproductive biology of these veronicellid species; they will help us to understand why they are such successful invaders and thereby to predict and prevent their further spread.

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Preface

This thesis is presented as two separate chapters. Each chapter is formatted as a manuscript that will be published in a peer-reviewed journal. This preface provides a description of the contribution my collaborators provided and the rationale behind each manuscript's authorship. I will be the first author for both manuscripts as I completed most of the work and have written the entire thesis. The first chapter and second chapter will be co-authored by Robert Cowie, my graduate advisor, as he provided guidance in experimental design and writing. The second manuscript will include Kirsten Poff and Gordon Bennett as authors. Gordon provided insight, laboratory space, and supplies necessary to complete this project. He also assisted with experimental design. Kirsten helped me to complete laboratory work and data analysis. Her contribution was vital to the completion of this project.

Chapter 1. Invasive characters of slugs in the Hawaiian Islands and temperature response suggesting possible range shifts under a changing climate

Introduction

Invasive species are non-native organisms that establish readily and negatively impact native systems. They are the second greatest cause of recent extinctions (Bellard et al. 2016) and directly impact species listed as threatened or endangered under the U.S. Endangered Species Act (Pimentel et al. 2005). It is conservatively estimated that the United States alone loses $120 billion dollars annually because of the damage caused by invasive species (Pimentel et al. 2005). Island ecosystems and endemic biodiversity are particularly at risk from the effects of invasive species (Reaser et al. 2007; Gasc et al. 2010; Dueñas et al. 2018). With the aid of increased globalization, islands once too remote for non-native species to reach now face the continual threat of invasive species introductions. Invasive species, both intentionally and unintentionally brought to islands across the globe, are well documented for their role in native ecosystem destruction (e.g. Choi & Beard 2012; Courchamp et al. 2003; Davis et al. 2009; Hansen & Müller 2009; LaRosa et al. 2008; Loope et al. 1988; McNatty et al. 2009; Medina et al. 2011; St. Clair 2011).

Native island ecosystems are also threatened by climate change. For example, the climate of the Hawaiian Islands is likely to undergo significant change in the coming decades. Projections suggest changes in precipitation intensity and frequency, decreasing overall rainfall, and increasing land and sea temperatures (Giambelluca et al. 2008; Chu et al. 2010; Safeeq et al. 2013; Timm et al. 2015). Higher temperatures will decrease the diurnal temperature range (Giambelluca et al. 2008; Safeeq et al. 2013). This means invasive species once restricted to warm areas by cool nights, may be able to expand their ranges (Safeeq et al. 2013). For instance, higher elevations will also be more susceptible to invasion from species restricted to lowland environments as warming occurs (Giambelluca et al. 2008).

As climate change alters the Hawaiian Islands, it is also likely that the range of invasive molluscs will change, in some cases expand. Many have remained in low to middle elevations in the Hawaiian Islands, but some invasive species also occur in native habitats at higher elevations

(Cowie 1998, 2001; Meyer & Cowie 2010). Overwhelmingly, one of the most important issues regarding invasive molluscs is that once established, they are difficult to eradicate (Cowie 2001). While their impacts often go unstudied, invasive molluscs affect native malacofaunas through direct or indirect competition and predation (Race 1982; Rawlings et al. 2007; Holland et al. 2012). Invasive molluscs can have even broader ecosystem impacts such as directly shaping plant communities through herbivory of selected seedlings (Joe & Daehler 2008; Shiels et al. 2014). Molluscs may also be part of complex multi-species invasional meltdowns, altering native habitats and replacing native species (Meza-Lopez & Siemann 2015; O'Loughlin & Green 2016). Along with the effects invasive species have on native ecosystems, molluscan invaders pose economic risks to humans as agricultural pests and to human health as parasite vectors (Barker 2002; Carlsson et al. 2004; Kim et al. 2014; Pointier et al. 2007).

Determining characteristics of invasive species may be valuable for evaluating the risks posed by non-native species. However, identifying overarching characteristics that are associated with invasiveness across divergent taxa has been challenging (Kolar & Lodge 2002). Instead, careful examination of characteristics of successful invaders within a group may shed light on what makes an invader successful. Studies have identified fecundity (Naylor 1996; Cowie 1998; Keller et al. 2007), egg/juvenile size, adult size, reproductive potential, breeding system, and phylogenetic relatedness to known invasive species (Cowie et al. 2009) as the primary characteristics facilitating molluscan invasiveness. Life history traits in particular are thought to be key to their success (Carlsson et al. 2004; Cowie et al. 2009; Keller et al. 2007).

Three invasive species in the family Veronicellidae are currently established in the Hawaiian Islands: *Veronicella cubensis* (Pfeiffer 1840), *Sarasinula plebeia* (Fischer 1868), and *Laevicaulis alte* (Férussac 1822), as well as an unconfirmed report of *Diplosolenodes occidentalis* (Guilding 1825) (Kim et al. 2016). *Sarasinula plebeia* has only been found in one locality on O'ahu. *Veronicella cubensis* and *Laevicaulis alte* (Figure 1.1) are found on all six of the largest Hawaiian Islands (Kim et al. 2016). Most Veronicellidae are tropical and sub-tropical slugs (Barker 2001). Primarily nocturnal, veronicellids are active all year but especially in moist conditions (Hata et al. 1997). These slugs are typically found on the ground among leaf litter, under objects, or buried in the earth. Some species can have economic impacts (e.g. Rueda et al.

Figure 1.1 *Veronicella cubensis* (left) and *Laevicaulis alte* (right).

2002). Cowie et al. (2009) listed Veronicellidae among the top eight families of invasive molluscs that could have the greatest negative impact on the United States economy and environment.

Relatively little is known about the life history of Veronicellidae. Most species are oviparous, with a few exceptions of ovoviviparity and viviparity (Baker 1925; Barker 2001). The number of eggs per clutch may vary greatly among species (Barker 2001). Veronicellids are characterized as being ditrematous (Barker 2001). Mating is reciprocal and veronicellids may be protandric (Baker 1925; Barker 2001). Baker (1925) speculated that sexual maturity of veronicellids is dependent on the time of year more than on body size.

Understanding the life histories of invasive molluscs will inform strategies to mitigate the impact and spread of invasive species. This study provides initial insights into the life history traits of *V. cubensis* and *L. alte* that may influence their potential to be invasive, including sperm storage, self-fertilization, egg laying and mating behaviors, and the effect of temperature on growth rate, egg productivity, and hatching time.

Methods and Materials

Reproductive maturity, mating and egg laying

Nine adult *V. cubensis* were collected from a backyard in Kailua, Hawai‘i, on August 21, 2016. Three slugs were housed in each of three plastic containers (24 cm length x 14 cm width x 7 cm depth). These adults and the eggs and juveniles they produced were subsequently maintained following a similar protocol to that adopted by Capinera & Guedes Rodrigues (2015). Containers were filled with approximately 3 cm of Miracle Grow All Purpose Gardening Soil. These wild caught, parental generation slugs were kept at 22 ^{o}C, under 12 hours of artificial light per day and provided with an unlimited diet of romaine lettuce. A damp substrate was maintained by spraying the soil with water. Egg masses laid by the wild caught parents were removed as soon as the egg laying slug had abandoned the egg mass. Egg masses were placed in smaller plastic containers (13 cm length x 9 cm width x 7 cm depth) between two damp pieces of paper towel (Capinera & Guedes Rodrigues 2015). Containers with egg masses were kept under the same light and temperature conditions as the parents. After hatching, first filial generation (F1) juveniles were kept in the small container on damp paper towel and fed romaine lettuce. Before reaching two weeks of age three slugs from the clutch were randomly selected. One juvenile was housed as a singleton, the other two as a pair. All excess juveniles were killed. In total, 15 egg masses hatching from September 26, 2016 to November 6, 2016 were used, providing 15 singletons, and 15 pairs.

F1 juveniles housed as singletons and pairs were maintained on paper towel in small containers until they reached a length of about 3 cm, at approximately one month of age. F1 slugs were maintained under the same conditions as the parental generation. Once a day all slugs were located in their containers and their reproductive behavior was noted as either not active, mating, or laying eggs. To determine the length of time sperm could be stored, five of the 15 pairs were randomly selected to be separated and housed as individuals after their first copulation. All egg masses produced by F1 slugs were removed as soon as the parent slug left the egg mass. All singletons and pairs were kept until reaching 18 months of age, if they had not

died. Basic descriptive statistics were calculated to characterize behavior durations and their ranges.

The same procedure was followed for *L. alte*. Four slugs were collected from the University of Hawai‘i at Mānoa campus on August 21, 2016. However, the wild caught adults produced few egg masses. The resulting F1 juveniles proved difficult to maintain under laboratory conditions. Only four pairs and two singletons were successfully reared and only limited data could be collected on them.

Effects of temperature on juvenile growth, egg production, and hatching

Laevicaulis alte and *Veronicella cubensis* collected from the University of Hawai‘i at Mānoa campus as well as lab reared *V. cubensis* were maintained as described above for the parental generation with the exception that half were kept at 22 ^{o}C and other half 27 ^{o}C. These temperatures are similar to those used in other veronicellid rearing studies (e.g. Raut & Panigrahi 1988; Capinera & Guedes Rodrigues 2015). Egg masses were removed and housed in the same manner as before, at the same temperature as their parents.

The time from the parent slug concluding laying and leaving the egg mass until the first juvenile hatched was recorded as hatching time. After the first juvenile was recorded hatching from an egg mass a 24 hour period was allowed to pass. This gave a majority of the individuals a chance to hatch. After this first day all hatched juveniles were counted. If unhatched eggs remained, the eggs were checked once a day until all juveniles had emerged or no more juveniles emerged after a 24 hour period of time. The numbers of hatched juveniles and unhatched eggs were recorded for each egg mass.

After the first day of hatching, ten individuals from each egg mass were randomly selected to be weighed. All ten were weighed together to determine a mean weight. The ten selected slugs from an egg mass were housed together in a large plastic container on damp paper towel and maintained under the same conditions as were their parents. All other juveniles from the egg mass were killed. The sets of ten slugs were weighed every 14 days until reaching 6 months of age. Due to the difficulties of maintaining *L. alte* under laboratory conditions, only eight sets of

L. alte under cool conditions and only ten sets under warm conditions were successfully maintained for the entire 6 months. For *V. cubensis*, 22 sets were maintained under cool conditions and 15 in warm conditions. In both species, some individuals died before 6 months. The mean weight of the set of slugs was still recorded with the reduced number of individuals.

Over the course of 6 months, each set was weighed 14 times. The overall mean for all sets was calculated from the means of each set of ten siblings. This accounted for variation not only among siblings, but across offspring from different parents of the same species. To test whether temperature affected juvenile weight gain, a series of linear mixed effect models were fitted to the data using lme4 in R V3.5.0 (Bates et al. 2015; R Core Team 2018). Seven models were compared (Table 1.1), including models with a time effect, quadratic time effect, a temperature effect, and interactions between time and temperature and quadratic time and temperature. Weight data were normalized using a natural log transformation, base equals exp(1). The best fit model was distinguished using Akaike Information Criterion corrected for small sample size (AICc) (Akaike 1974) with the bbmle package in R V3.5.0 (Bolker 2017; R Core Team 2018). Data recorded on egg mass time to hatching, number of eggs per egg mass, and hatchability were gathered by species and temperature. The effect of temperature on hatchability was analyzed with a generalized linear model quasibinomial logistic regression in R V3.5.0 (R Core Team 2018). The effect of temperature on the total number of eggs was analyzed with a generalized linear model quasipoisson logistic regression in R V3.5.0 (R Core Team 2018). The days to hatching were analyzed for significant difference between temperatures with a Cox Proportional Hazard model (Cox 1972). All slugs were weighed individually at the end of the 6 month experiment. Individual weights were used to assess ranges in weight among siblings and within species.

Table 1.1 Mixed-effects regression models evaluated for *V. cubensis* and *L. alte*. Models with Time and Time2 accounted for a quadratic effect of time. Mass refers to juvenile weight, temperature is the treatments at 22 ºC or 27 ºC and (1|ID) is a random effect in which ID refers to a single set of slugs. Scale in R V3.5.0 centers each variable's column and then divides by the standard deviation. Model 7 is the null model.

Model	
Model 1	log(mass)~scale(Time)+scale(Time2)+Temperature+Temperature:scale(Time)+Temperature:scale (Time2)+(1|ID)
Model 2	log(mass)~scale(Time)+scale(Time2)+Temperature+(1|ID)
Model 3	log(mass)~scale(Time)+scale(Time2)+(1|ID)
Model 4	log(mass)~scale(Time)+Temperature+Temperature:scale(Time)+(1|ID)
Model 5	log(mass)~scale(Time)+Temperature+(1|ID)
Model 6	log(mass)~scale(Time)+(1|ID)
Model 7	log(mass)~1+(1|ID)

Results

Reproductive maturity, mating and egg laying

Among the 15 F1 *V. cubensis* housed as singletons, there were no recorded instances of self-fertilization, as assessed by the absence of any egg laying. On one occasion a single *L. alte* laid a fertilized egg mass from which juveniles hatched. This slug was initially housed as a pair, but its partner died long before the egg mass was laid and no mating had been recorded. This suggests this slug was not storing sperm, but that it had self-fertilized.

One of the five *V. cubensis* pairs selected to be split after their first copulation, never mated. Of the four pairs that did mate, only three pairs (six individuals) laid eggs (Figure 1.2). The mean time between mating and the final egg mass being laid was 137 days (Table 1.2). One individual, at 270 days, laid a gelatinous string with a few small eggs. These eggs were probably not fertilized as they showed no evidence of embryo development and quickly rotted. Individuals laid a mean of 4.2 egg masses (Table 1.2). The frequency of egg laying was about one egg mass per month. The longest time between egg laying was three months. By the end of the experiment, no split pair individuals had laid egg masses for at least 5 months.

Figure 1.2 *Veronicella cubensis* mating pair (top). *V. cubensis* laying eggs (bottom).

Of the 15 *V. cubensis* pairs, 13 mated during the 18 months of the experiment. The mean age at first mating was 203 days (Table 1.2). The earliest mating occurred at 126 days. The youngest individual to lay

eggs did so at 146 days (Table 1.2). The mean age at the first egg laying event was 226 days. Individuals housed as pairs were not distinguished from each other, and data for age at first egg laying were only collected if both individuals in a pair laid eggs at the same time or in close succession so that it was clear that both individuals were laying their first egg mass.

In total, 29 mating events were recorded among the 13 pairs of *V. cubensis* that mated. The mean duration over which mating behaviors were recorded was 1.5 days (Table 1.2). One F1 pair of *L. alte* was recorded as possibly mating on one day. All wild caught parent *L. alte* that did mate, did so within one day.

Among the ten *V. cubensis* pairs that remained together over 18 months, individuals in six pairs laid eggs. The mean duration of egg laying behavior for all egg laying slugs (both pairs and separated individuals) was 2.0 days (Table 1.2). The mean number of egg laying events per pair over the duration of 18 months was 8.0 (Table 1.2). Pairs laid multiple eggs masses per mating event. For every mating event approximately 3.2 egg masses were laid (Table 1.2).

Table 1.2 *Veronicella cubensis* life history data including reproductive maturity, mating and egg laying durations, sperm storage.

Behavior/Data type	**Mean ± standard deviation**	**Median**	**Range**
Sperm storage duration	137 ± 40 days	147 days	57-179 days
Egg laying events per individual using stored sperm (split pairs)	4.2 ± 1.5 events	4.0 events	2-6 events
Mating duration (pairs)	1.5 ± 0.6 days	1 day	1-3 day
Egg laying duration (pairs and split pairs)	2.0 ± 0.8 days	2 days	1-4 day
Egg laying events per pair	8.0 ± 0.8 events	8 events	7-9 events
Egg laying events per mating event (per pair)	3.2 ± 1.0 events	3.3 events	1.4-4.5 events
Age at first mating event (pairs and split pairs)	203 ± 42 days	199 days	126-305 days
Age at first egg laying event (pairs and split pairs)	226 ± 52 days	218 days	146-326 days

Effects of temperature on juvenile growth, egg production, and hatching

Figure 1.3. Newly hatched *Laevicaulis alte* (left) and *Veronicella cubensis* (right).

Model 1, which included a quadratic effect of time, temperature and a temperature time interaction, was the best fit to the growth data for *V. cubensis* juveniles (Tables 1.1 and 1.3). An estimate of 0.121 (SE = 0.016, 95% CI = 0.089, 0.153) indicates temperature does affect *V. cubensis* growth (Table 1.4 and Figure 1.4). The estimate of the time2:temperature interactions (estimate = -0.120, SE = 0.016, 95% CI = -0.152, -0.089) and time:temperature interactions (estimate = 0.096, SE = 0.016, 95% CI = 0.064, 0.128) suggest rate of growth differs between the two temperatures over time (Table 1.4). For *L. alte* the best fit models were Model 1 and Model 2 (Tables 1.1 and 1.3). According to Model 1, a cooler temperature causes a faster weight gain for *L. alte* juveniles (estimate = -0.051, SE = 0.023 95% CI = -0.101, -0.005). The estimate for temperature in Model 2 is -0.051 (SE = 0.024, 95% CI = -0.099, -0.003) and like Model 1 indicates a slight increase in the growth of juveniles in the cooler temperature (Table 1.4 and Figure 1.4). The major difference between these two models is that Model 1 includes a temperature time interaction and Model 2 does not. The presence of this interaction in one model and not the other suggests that the rate at which temperature is effecting weight gain over time is fairly similar between the two temperatures with just minor differences.

Individual weights at 6 months reveal that at both temperatures the weight range among *V. cubensis* siblings can be large, just over 8 g at 22 °C and just over 7 g at 27 °C (Table 1.5). Both temperature conditions yielded an average sibling weight range of just over 3.5 g. Maximum sibling weight ranges at 6 months were smaller for *L. alte* than for *V. cubensis*; however, the maximum weight range of *L. alte* at 22 °C was nearly twice that at 27 °C.

A five degree temperature difference resulted in a significant difference in time to hatching for both species (Table 1.6). At 27 ^{o}C *V. cubensis* mean time to hatching was four days less than

at 22 ºC and for *L. alte* the difference was six days. Temperature did not significantly affect the number of eggs per egg mass or the percent hatchability in either species (Tables 1.7 and 1.8).

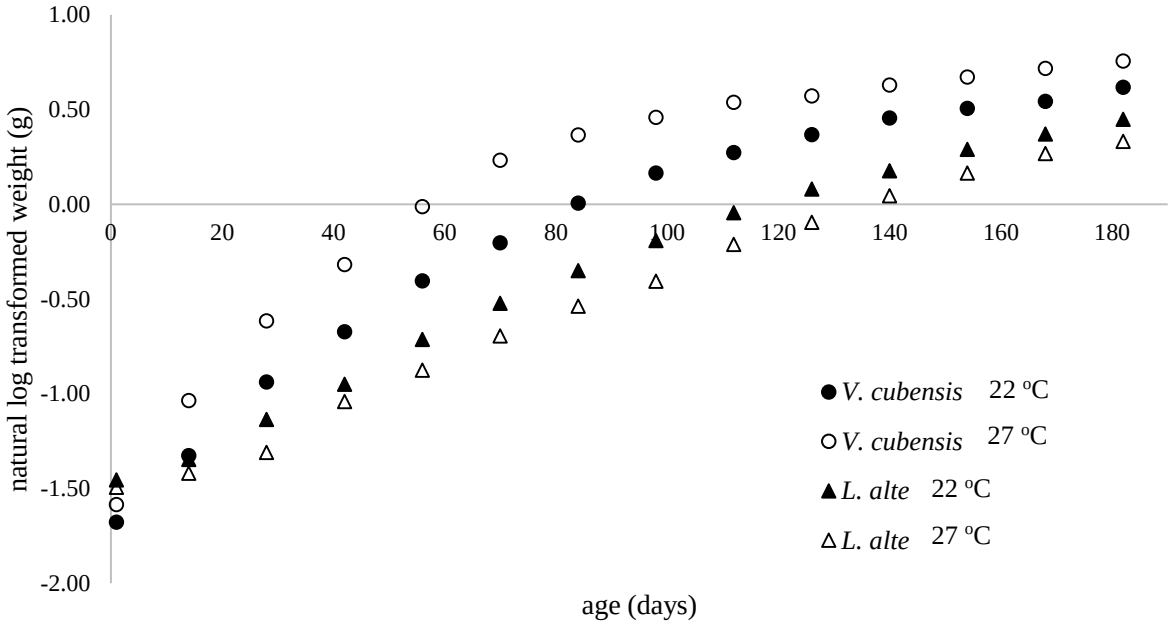


Figure 1.4 Mean weight of *V. cubensis* and *L. alte* sets in 22 ºC and 27 ºC from hatching until 6 months of age. At both temperatures *V. cubensis* weighed about 0.02 g upon hatching and *L. alte* weighed about 0.03 g. At six months the average *V. cubensis* at 27 ºC was 5.69 g and at 22 ºC was 4.13 g. The average *L. alte* at 27 ºC at six months was 2.14 g and 2.80 g at 22 ºC.

Table 1.3 Mixed-effects regression evaluated with AICc.

Model	AICc score *V. cubensis*	delta AICc score *V. cubensis*	df *V. cubensis*	AICc score *L. alte*	delta AICc score *L. alte*	df *L. alte*
Model 1	80.9	0.0	8	82.4	0.0	8
Model 2	152.8	72.0	6	86.7	4.3	6
Model 3	188.6	107.7	5	91.1	8.7	5
Model 4	1007.2	926.3	6	150.9	68.5	6
Model 5	1010.7	929.8	5	151.2	68.7	5
Model 6	1046.4	965.5	4	155.9	73.5	4
Model 7	2018.5	1937.7	3	956.1	873.7	3

Table 1.4 Best mixed-effects models of juvenile growth. The models were selected using AICc (Table 1.3). The model for *V. cubensis* is the best fit model (Model 1). The models for *L. alte* are the first and second best fit models (models 1 and 2, respectively).

Species	Model				
V. cubensis	log(mass)~scale(Time)+scale(Time2)+Temperature+Temperature:scale(Time)+Temperature:scale (Time2)+(1|ID)				
	effect	**estimate**	**standard error**	**95% confidence intervals**	**t-value**
	Time	1.219	0.392	(0.450, 1.987)	3.116
	Time2	0.821	0.391	(0.053, 1.589)	2.100
	Temperature	0.121	0.016	(0.089, 0.153)	7.714
	Time:Temperature	0.096	0.016	(0.064, 0.128)	5.974
	Time2:Temperature	-0.120	0.016	(-0.152, -0.089)	-7.438
	intercept	-3.101	0.379	(-3.865, -2.338)	-8.167
L. alte	log(mass)~scale(Time)+scale(Time2)+Temperature+Temperature:scale(Time)+Temperature:scale (Time2)+(1|ID)				
	Time	3.176	0.582	(1.980, 4.373)	5.223
	Time2	-1.694	0.608	(-2.881, -0.507)	-2.808
	Temperature	-0.053	0.023	(-0.101, -0.005)	-2.295
	Time:temperature	-0.050	0.024	(-0.098, -0.002)	-2.081
	Time2:temperature	0.047	0.024	(-0.0002, 0.094)	1.956
	intercept	0.139	0.582	(0.186, 0.360)	0.239
	log(mass)~scale(Time)+scale(Time2)+Temperature+(1|ID)				
	Time	1.917	0.060	(1.797, 2.036)	31.573
	Time2	-0.519	0.060	(-0.638, -0.400)	-8.607
	Temperature	-0.051	0.023	(-0.099, -0.003)	-2.214
	intercept	0.096	0.584	(-1.106, 1.290)	0.166

Table 1.5 Weight variation among sibling and within species at the end of the experiment when each slug was weighed individually.

Species & Temp. (ºC)	Average weight range (g) among siblings	Maximum weight range (g) among siblings	Average weight± standard deviation (g)	Median weight (g)	Range (g)
V. cubensis 22	3.62	8.38	4.21±1.60	4.19	0.66-9.04
V. cubensis 27	3.88	7.30	5.70±1.92	5.49	2.25-11.39
L. alte 22	2.68	6.48	2.65±1.60	2.30	0.39-7.69
L. alte 27	1.57	3.82	2.04±0.86	1.84	0.73-5.02

Table 1.6. Time to hatching in days for *V. cubensis* and *L. alte* at 22 ºC and 27 ºC, considered from the day the parent left the egg mass until the first juveniles emerged from the eggs. A Cox Proportional Hazard Model Likelihood Ratio Test determined the significance of temperature on hatching ($p \leq 0.05$).

Species & temperature (ºC)	Mean ± standard deviation (days)	Median (days)	Range (days)	p-value
V. cubensis 22	16.9 ± 1.7	17	14-23	2×10^{-12}
V. cubensis 27	12.7 ± 1.5	13	9-16	
L. alte 22	21.4 ± 1.9	21.5	18-25	3×10^{-10}
L. alte 27	15.7 ± 1.0	16	14-17	

Table 1.7. Number of eggs per egg mass for *V. cubensis* and *L. alte* at 22 ºC and 27 ºC. Total number of eggs per egg mass was determined by counting the number of hatched juveniles plus the number of unhatched eggs after juveniles stopped emerging. Data for *V. cubensis* at 22 ºC encompass egg masses from both the temperature study and the life history study. Percent hatching for *V. cubensis* at 22 ºC includes 7 extra egg masses in which the total number of eggs was not counted but in which no eggs hatched, thus 0% hatching.

Species & temperature (ºC)	n	Mean ± standard deviation (eggs)	Median (eggs)	Range (eggs)	Average % hatching
V. cubensis 22	71	67 ± 31	64	6-197	74
V. cubensis 27	16	66 ± 17	68.5	31-94	93
L .alte 22	17	49 ± 36	39	12-142	90
L .alte 27	13	39 ± 18	32	22-83	84

Table 1.8 Results of generalized linear models regarding the impact of an increase of temperature on egg hatchability and the total number of eggs per egg mass. Hatchability was analyzed using a quasibinomial logistic regression and total number of eggs with a quasipoisson logistic regression.

Species & characteristic	Estimate	95% confidence interval	Standard error	p-value
V. cubensis hatchability	0.8669	(-0.117, 2.134)	0.5594	0.125
L. alte hatchability	-0.381	(-1.761, 1.043)	0.695	0.588
V. cubensis total number of eggs	-0.024	(-0.268, 0.206)	0.120	0.837
L. alte total number of eggs	-0.246	(-0.7531, 0.2394)	0.2519	0.336

Discussion

Prior to this study, little was known of the life history of *Laevicaulis alte* and almost nothing was known of the life history of *Veronicella cubensis*. Both are common invasive species and pose economic and health risks. By examining life history characteristics such as mating, egg laying, age at reproductive maturity, sperm storage, number of eggs per egg mass, hatchability, and juvenile growth we are able to begin to understand why these species are successful invaders and plan effective methods to prevent their spread, particularly in the face of climate change.

Life history characters with implications of successful colonization

Reaching reproductive maturity at a young age in the context of a long lifespan is a life history character that potentially aids invasiveness. The earliest recorded mating by *V. cubensis* occurred at 126 days, about 4 months, and the average was 203 days, closer to six months (Table 1.2). Juvenile *V. cubensis* housed in boxes with ten slugs at 27 ºC were observed mating and laying eggs before the end of the six month experiment. Since weight is gained faster in warmer temperatures it is likely that reproductive maturity is reached faster. Other veronicellids reach reproductive maturity around the same age as *V. cubensis*: *Diplosolenodes occidentalis* near seven months, *Leidyula moreleti* at six months, and *Sarasinula plebeia* at six months or when the slug reaches a weight of 3 g (Caballero et al. 1991), or as early as 3-4 months (Rueda Pinzon 1989). Although only 4 of the 45 experimental *V. cubensis* individuals, housed as either singletons or pairs, had died by the end of the 18 month experiment, it was nonetheless possible to obtain a minimum estimate of longevity. A few of the wild caught parents, which were reproductively mature when captured, were maintained for 18 months and a singleton was kept until 24 months of age. Furthermore, *Sarasinula plebeia* is estimated to live at least two and possibly up to four years (Caballero et al. 1991) and *Leidyula floridana* lives past 18 months (Capinera & Guedes Rodrigues 2015). It is thus likely that *V. cubensis* regularly lives for 2 or more years. If slugs reach reproductive maturity around 6 months of age, this means they have 18 months of life in which they may be reproductively active. Over the course of the study, *V. cubensis* pairs laid an average of eight egg masses (Table 1.2). Assuming each slug in the pair

laid four egg masses, the rate of productivity is about 4 egg masses per year, which is similar to that of *Sarasinula plebeia* (Caballero et al. 1991). The average egg mass at 27 ºC contained 66 eggs. Therefore, a single slug is capable of producing nearly 400 eggs that hatch with 93% success in 18 months of reproductively mature life. Thus, a small number of individuals can quickly proliferate facilitating rapid colonizing of a new location.

The duration of mating for *Veronicella cubensis* is remarkably long and may even leave the slug vulnerable. Baur (1998) commented on the possibility of molluscan mating occurring for up to 36 hours. Long duration mating is surprising in slow-moving molluscs as the longer time spent in one place would subject the individuals to risk of predation and perhaps desiccation. It is also likely that the longer two individuals are in contact with one another the greater is the potential for parasite exchange (Baur 1998). The freshwater snail *Pomacea canaliculata* may mate for nearly 20 hours in order to exchange adequate quantities of sperm to fertilize all the eggs produced by the female (Burela & Martín 2011). Since sperm can be stored up to six months in *V. cubensis,* it is possible this species requires long mating durations to exchange large amounts of sperm. However, it is also possible that long duration mating evolved as a mechanism to cope with sperm competition (Klemme & Firman 2013). On one occasion three individuals of *V. cubensis* were found in a mating triplet (Figure 1.5). The multiple slug mating lasted two days. This behavior of multiple slug mating is well recorded in *Veronicella sloanii* (Clarke & Fields 2013) and *Sarasinula plebeia* (Rueda Pinzon 1989). However, *V. sloanii* has a much shorter mating duration, 0.4-2 hours (Clarke & Fields 2013) and *S. plebeia* an average of 0.5 hours (Rueda Pinzon 1989). Mating in *Leidyula floridana* takes on average 6 hours (Rueda Pinzon 1989). Similarly, *L. alte* has a short mating duration. In this study, *L. alte* pairs were never recorded mating for more than one consecutive day. This observation aligns with a previous *L. alte* study that found mating duration to be 30-60 minutes (Nagabhushanam & Kulkarni 1971). It should be noted that in the present study behavioral observations were only recorded once a day for each

Figure 1.5. Three *Veronicella cubensis* simultaneously mating.

slug. The data collected on behaviors can only be interpreted in days, not exact hours. Further work should be done to clarify a relationship between mating duration and successful sperm transfer.

Similar to the duration of mating in *V. cubensis*, that of egg laying was long. Laying of a single egg mass tended to occur over a two day period, which is similar to the time taken by *Leidyula floridana* (Capinera & Guedes Rodrigues 2015). *Veronicella ameghini* takes approximately 45 minutes to lay each egg, 3-15 eggs in total (Tompa, 1980). This time per egg roughly corresponds to the rate of an average number of eggs laid by *V. cubensis* (Table 1.6) over two days. As only sparse observations could be made on *L. alte* egg laying, it is assumed that *L. alte* lays eggs within one day. This inference is supported by Nagabhushanam & Kulkarni (1971) who reported that *L. alte* lays 46-70 eggs at a rate of 15 to 20 minutes per egg. *Laevicaulis alte* may benefit from completing mating and egg laying within a day whereas the long duration behaviors seen in *V. cubensis* place an increased risk on the slug. *Veronicella cubensis*, on average, lays nearly double the number of eggs per egg mass than does *L. alte* (Table 1.7) and twice the number laid by *Sarasinula plebeia*, which also has a much shorter mating duration (Rueda Pinzon 1989). There appears to be a tradeoff between high risk long duration behaviors and producing more offspring in one event.

Sperm storage and self-fertilization are well known phenomena in terrestrial gastropod species (e.g. Selander & Kaufman 1973; Baur & Baur 2000; Rogers & Chase 2002; Chase & Darbyson 2008; Kupfernagel et al. 2013). Among the reproductive characters that may facilitate *V. cubensis* being a successful invader, sperm storage may be important. The data suggest *V. cubensis* routinely relies on stored sperm to produce fertilized eggs, even when near a potential mate. Because there were no recorded instances of self-fertilization in *V. cubensis* the use of autosperm competing or being used in conjunction with allosperm can be ruled out. This study suggests that sperm can be stored for almost 6 months (Table 1.2). Thus, an isolated individual could use stored sperm to successfully produce offspring and colonize new areas. Duration of sperm storage has yet to be studied in *L. alte*; however, self-fertilization was observed and is common in other veronicellids (Rueda et al. 2002).

Effects of temperature with implications for range expansion

While temperature influencing growth rate (Table 1.4 and Figure 1.4) and time to hatching (Table 1.6) is unsurprising it may be important when considering the potential range expansions of invasive species, especially in the face of climate change. The Hawaiian Islands will become hotter and drier (Chu et al. 2010; Giambelluca et al. 2008; Timm et al. 2015). Currently, both *L. alte* and *V. cubensis* occur on both the wetter windward and drier leeward sides of the islands; *L. alte* extends up to 488 m and *V. cubensis* up to 1,203 m above sea level (Kim et al. 2016). Their current elevational ranges are probably limited by temperature. Average temperatures in the highest elevation areas, particularly on the wetter windward sides of the islands, rarely exceed 20 ^{o}C (Giambelluca et al. 2014). If veronicellid eggs do not hatch or take a very long time to hatch at 10 ^{o}C or 15 ^{o}C (Raut & Panigrahi 1988; Rueda Pinzon 1989), they could not produce offspring in these cooler areas. As warming occurs these cool temperatures will no longer restrict invasive range expansions (Giambelluca et al. 2008). Drier conditions may also alter current ranges. Eggs of both *L. alte* and *V. cubensis* are gelatinous and at risk of desiccation with prolonged exposure to dry conditions. Adult *V. cubensis* also may risk desiccation from long duration mating and egg laying. In India, Nagabhushanam & Kulkarni (1971) found *L. alte* reproduction coincided with the rainy season. If precipitation occurs less often but warmer temperatures promote shorter times to hatching (Table 1.6), both species may possibly still produce young because a decreased time to hatching would mean a decrease in the time the eggs are exposed to drying conditions.

Other veronicellids also demonstrate similar trends of temperature dependent hatching times. Raut & Panigrahi (1988) found that *L. alte* eggs took 20.9 days to hatch at 20 ^{o}C, 17.5 days at 25 ^{o}C, and 13.3 days at 30 ^{o}C, while failing to hatch at 10 ^{o}C, 15 ^{o}C, and 35 ^{o}C. *Veronicella ameghini* eggs at 20 ^{o}C hatch in 20 days (Tompa, 1980) and *Leidyula floridana* eggs at 26 ^{o}C in about 14 days (Capinera & Guedes Rodrigues 2015). *Sarasinula plebeia* failed to hatch at 15 ^{o}C and took >30 days at 20 ^{o}C, ca. 17 days at 25 ^{o}C and ca. 13 days at 39 ^{o}C (Rueda Pinzon 1989). Furthermore, if *V. cubensis* juveniles gain weight more readily and reach reproductive maturity sooner, because of warmer temperatures, they may potentially produce more eggs throughout

their lifetime. Additional studies should be performed to determine a correlation between weight and reproductive success. Even though *V. cubensis* reaches reproductive maturity near 6 months of age, the slugs are still growing (Table 1.2). The average one year old *V. cubensis* weighed 8.47 g and one slug, at 18 months old, weighed 30 g. *Laevicaulis alte* similarly appears to reach weights much greater than those recorded at 6 months. One wild caught *L. alte* weighed about 11 g. The increased rate of weight gain of *L. alte* at 22 ^{o}C compared to 27 ^{o}C was unexpected. Although we remain skeptical of these data because of the small sample size and the difficulty of maintaining *L. alte* in laboratory conditions, which may have been related to diet (cf. Rueda Pinzon 1989), there is a possibility that these temperature effects may reflect unknown physiological or competition driven characters. Climate change may lead to expansion or contraction of the ranges of *V. cubensis* and *L. alte* in the Hawaiian Islands and elsewhere. Growth and hatching will be affected by a warming climate and therefore impact the success of these invasive species.

Conclusion

This study describes and quantifies important aspects of the life histories of two invasive slug species in Hawai‘i that may be relevant to their invasiveness. By beginning to understand the effect of temperature on these aspects, this information is important for developing appropriate pest management strategies that can be employed to predict and prevent the spread of both species in new areas and in the face of a changing climate. As both *V. cubensis* and *L. alte* pose economic and health risks it is important that further research be undertaken.

Chapter 2. Intergenerational microbiome profiling suggests extracellular transmission of the hindgut microbiome community in invasive veronicellid slugs

Introduction

In contrast to other animal groups for which considerable information about the gut microbial communities is available (e.g. Schmitt-Wagner et al. 2003; Sommer & Bäckhed 2013; McLaughlin et al. 2015), the gut microbiota of terrestrial molluscs has received little attention. From the studies that have been done, there is evidence that terrestrial molluscan gut bacterial communities readily change with alterations in diet (Cardoso et al. 2012) and differences in habitat and physiological state (Nicolai et al. 2015). Phylogenetically closely related species with similar ecology can exhibit highly complex and diverse gut microbe communities (Van Horn et al. 2012). Previous microbiome studies on the invasive giant African snail, *Lissachatina fulica* (Cardoso et al. 2012), and on planorbid snails (Van Horn et al. 2012) have been largely motivated by the damaging economic and human health impacts of these species. Invasive molluscs have been estimated to cost the United States $120 billion dollars annually (Pimentel et al. 2005). Research on the microbiome of molluscan pests, associated with their physiology and behavior, may provide powerful insights relevant to more effective prevention and control.

Veronicella cubensis (Pfeiffer 1840), originally from the Caribbean, is a common invasive systellommatophoran slug species in the family Veronicellidae. This slug is a widespread and abundant garden and horticultural pest in Hawai‘i (Hata et al. 1997; Kim et al. 2016) and elsewhere in the Pacific Basin (Robinson et al. 2009). It is also capable of hosting the nematode *Angiostrongylus cantonensis*, the most common causative agent of eosinophilic meningitis in humans (Kim et al. 2014). Due to the understudied nature of terrestrial mollusc gut microbes, characterizing the microbial communities of the widely invasive *V. cubensis* is of interest. In particular, the egg laying behavior of *V. cubensis* and some other veronicellid slugs (Capinera & Guedes Rodrigues 2015) provides an opportunity to address the possible transmission of the gut microbiome from parent to offspring via an extracellular substance, an unexplored aspect of terrestrial molluscan life histories.

Among invertebrates there is great diversity in bacterial transmission methods. A number of species vertically transfer microbes that are usually required for development and host fitness directly into developing embryos. For example, brooded *Corticium* sp. sponge embryos harbor microbial symbionts of their parents (Sharp et al. 2007) and aphids form specialized bacteriocyte cells during embryological development and subsequently acquire parental microbes (Braendle et al. 2003). Other organisms adopt extracellular pathways such as using substances to transfer the microbes. For example, plataspid stinkbugs place symbiont capsules on their eggs and in order to successfully transfer the microbes, offspring must ingest the contents of the capsule (Fukatsu & Hosokawa 2002). To create a new successful colony, some species of termites with specialized fungal symbionts carry fungal conidia in a bolus in the foregut to inoculate a new colony (Johnson et al. 1981). Necrophagous burying beetles inoculate animal carcasses with oral and anal microbial secretions that change the carcass's microbial community and probably allow the transmission of important microbiota from adult to larvae (Shukla et al. 2018). Strong similarities are found between parental and larval gut bacterial communities in the dung beetle, *Euoniticellus intermedius*, a species that provides a "maternal gift", probably fecal material, to the brood ball (Shukla et al. 2016).

Figure 2.1 *Veronicella cubensis* laying eggs (top). Egg mass with brown substance (bottom).

While laying eggs *V. cubensis* circles clockwise depositing a string of eggs (Figure 2.1). Eggs are laid from the female pore, which is just under the notum, approximately half way down on the right side of the body. An average egg mass contains 66 eggs (Sommer, M.S. thesis chapter 1), which are connected by a mucous string. At the end of the coiled egg string, where no more eggs are added, the string thickens and is wrapped around the egg mass. This structure keeps

the egg mass intact. On every egg along the string a thread-like brown substance is deposited as the eggs are being laid (Figure 2.1). When describing the egg masses of *Leidyula floridana*, Capinera & Guedes Rodrigues (2015) stated that this substance "appeared to be fecal matter but much drier and thinner than typical fecal matter." This describes well the material deposited by *V. cubensis*. Since a number of invertebrates are known to transfer essential microbes to their offspring via extracellular methods I hypothesize that the brown substance laid on *V. cubensis* and other veronicellid slug eggs is a specialized form of fecal material that might provide a mechanism to vertically transfer gut microbes from parent to offspring through direct contact upon hatching. To test this hypothesis, we characterized the bacterial communities of (i) the hindgut of adult *V. cubensis* (ii) the brown substance (iii) juvenile *V. cubensis* hatched from surface sterilized eggs and (iv) juvenile *V. cubensis* hatched from eggs with brown substance and not surface sterilized. These communities were then compared to one another to determine the origin of the brown substance and understand the potential origin of the microbiome associated with juvenile *V. cubensis*.

Methods and Materials

Sampling

Veronicella cubensis adults were collected from the University of Hawai'i at Mānoa campus in the spring of 2017. Slugs were immediately housed in plastic containers (24 cm length x 14 cm width x 7 cm depth). Four or five slugs were placed in each container. Containers were filled with approximately 3 cm of Miracle Grow All Purpose Gardening Soil. These wild caught slugs were kept at 22 ^{o}C, under 12 hours of artificial light per day and provided an unlimited diet of romaine lettuce. When a slug started to lay eggs, the egg laying slug and its egg mass were gently picked up and moved from the large communal container to a smaller plastic container (13 cm length x 9 cm width x 7 cm depth) with 1 cm of soil from the communal container. Egg laying takes approximately two days (Sommer, M.S. thesis Chapter 1) so the egg laying slug was checked once a day until it was no longer attached to the egg mass. Slugs were not provided any

food during or after egg laying and therefore starved for two or more days prior to sampling. This was undertaken to reduce the influence of environmental and food-borne microbes on downstream analyses. The eggs were removed from the container and the brown substance was collected from the egg mass using sterilized forceps and aseptic techniques. Then the slug was killed with boiling water and immediately placed in 95% ethanol. Adult slugs were dissected to remove an approximately 1 cm long part of the most posterior portion of the hindgut. As an environmental control, three soil samples were taken from each of the two communal containers that produced egg laying slugs. Three hindgut samples were also taken from wild caught adult *Laevicaulis alte*, another non-native veronicellid species found in the Hawaiian Islands that does not deposit a brown substance on its egg masses. Sampling *L. alte* served as a comparison between two ecologically similar invasive slugs with different egg laying behaviors.

To obtain juvenile *V. cubensis*, additional egg masses laid by the remaining wild caught slugs were used. Immediately after deposition an egg mass was gently split in half. One half was left as is, with brown substance and soil particles, and placed in a small container between two damp pieces of paper towel. The other half was cleaned of brown substance and soil particles under sterile conditions. The cleaned half was placed in a small container between damp paper towels for one week. After one week, the eggs were surface sterilized by three washes of 10% bleach. It was necessary to wait a week before sterilization because eggs washed with sterilizing substances such as bleach or ethanol immediately after being laid never hatched. The washed eggs were placed in a new small sterile container in paper towel. Both halves of an egg mass were left alone until they hatched. Once juveniles emerged they were immediately placed in 95% ethanol.

Getting juveniles to hatch from surface sterilized eggs was challenging. Waiting one week before surface sterilizing the eggs allowed visual confirmation that the embryos were developing but may have allowed microbes to pass through the gelatinous egg membrane prior to sterilization. Also, instead of using part of the posterior portion of the juvenile hindgut, as for the adults, entire juvenile bodies were used because of their small size. Hatchling *V. cubensis* are approximately 1 cm long and weigh about 0.02 g (Sommer, M.S. thesis chapter 1). This is

similar to the small portion of the adult hindgut used. By using whole juvenile slugs I was sampling not only the internal microbes but also microbes on the exterior surface of the body.

The following samples were collected and stored at -20 ^{o}C until processing: five *V. cubensis* hindgut samples with their five corresponding brown substance samples, six soil samples from *V. cubensis* containers, three *L. alte* hindgut samples, a single *L. alte* soil sample, five *V. cubensis* juveniles hatched from eggs with brown substance and five hatched from surface sterilized eggs.

DNA was extracted using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). All samples of juveniles required manual maceration with a sterile pestle to increase DNA yields. A negative sequencing control was included, using the same protocols as for the samples to ensure there was no kit or lab contamination. Extracted DNA was quantified using a Qubit 3.0 fluorometer (Thermofisher). Samples were then sent to SeqMatic (Fremont, California, USA) for PCR amplification of the bacterial 16S rRNA gene, library preparation, sequencing, and the creation of a community profile. SeqMatic performed sequencing using an Illumina Miseq with 500 cycles (2x250 paired-end reads) and V2 chemistry. Qiime V1.9.0 and GreenGenes 16S Database (13_8) (Caporaso et al. 2010) were used to generate a bacterial community profile with a 97% species match threshold. When possible, bacterial operational taxonomic units (OTUs) were identified to species level.

Analysis

The presence and abundance of bacterial OTUs in each sample were determined from read counts and proportions of reads. Ten samples with low read counts were removed from the analysis (see Table 2.1). The data were rarefied to 10,079 reads to include as many samples as possible while retaining a large amount of the read information. A rarefaction curve was produced to determine OTU saturation using the vegan package in R (Oksanen et al. 2018; R Core Team 2018). Shannon diversity was calculated for each sample before and after rarefaction, also using the vegan package. Bray-Curtiss dissimilarity was determined to be the best method of beta diversity analysis for the data, using the vegan function rankindex in R V3.5.0 (Oksanen et al. 2018; R Core Team 2018).

A non-metric multidimensional scaling plot (NMDS) was implemented in R V3.5.0 (R Core Team 2018) based on sample type: hindgut, brown substance, soil, and juveniles from eggs with brown substance. The resulting sample type clusters were analyzed for significant dissimilarity using an analysis of similarity (ANOSIM) with 999 permutations (Clarke 1993) and for significance of variance strength using a non-parametric multivariate ANOVA (Anderson 2001) with the adonis function in the vegan package in R V3.5.0 (Oksanen et al. 2018; R Core Team 2018). Both of these analyses were run on both binary and Hellinger transformed data (Legendre & Gallagher 2001).

To display the abundance of bacterial taxa and the relationships among sample types, a heatmap with dendrograms was created based on Bray-Curtis dissimilarity. All OTUs representing greater than 5% of total read counts were included. Sequences of the bacterial OTUs identified as possible components of the adult slugs' hindgut core microbiome were verified using NCBI-BLASTN (Altschul et al. 1997).

Because the negative control showed low-level contamination (9,999 reads) the above analyses were also run using a data set in which read counts from each OTU in the negative control were subtracted from read counts of these OTUs in all other samples. Results from the two data sets were nearly identical. Furthermore, the OTUs in the negative control were either not found in the other samples or found at much lower read counts, indicating that the contamination was limited to the negative control. Thus, all presented results and discussion include unfiltered data sets.

Results

Soil and brown substance samples contained the highest diversity of bacteria with an average of 468 and 352 OTUs, respectively (Table 2.2). *Veronicella cubensis* and *L. alte* hindguts and the juveniles from eggs with brown substance contained fewer bacteria (189, 190, and 141 OTUs, respectively) and the juveniles from surface sterilized eggs averaged only 49 OTUs (Table 2.2). The NMDS plot represents the five sample types: soil, hindgut (both *V. cubensis* and *L. alte*), brown substance, juveniles hatched from eggs with brown substance, and juveniles hatched from

surface sterilized eggs (Figure 2.2). The juveniles from eggs with brown substance overlap all other sample types. The brown substance cluster and a portion of the soil cluster sit within the hindgut cluster. The three juvenile from surface sterilized eggs samples, although unable to form an ellipse, partly overlap with the juveniles from eggs with brown substance but not with the other sample types. ANOSIM (binary: $R = 0.6696$, $p \leq 0.001$ and Hellinger: $R = 0.7425$, $p \leq 0.001$) and adonis (binary: $p \leq 0.001$ and Hellinger: $p \leq 0.001$) suggest that all sample types are distinct from one another and that individual samples within each sample type more closely resemble one another than individuals from other sample types.

The heatmap (Figure 2.3) shows that the soil samples, which all cluster together, contain a high bacterial diversity. The hindguts (*V. cubensis* and *L. alte*) and brown substance (from *V. cubensis*) cluster together and all contain a large proportion of three different Gammaproteobacteria, i.e. *Cronobacter dublinensis* and OTUs identified as members of families Enterobacteriaceae and Aeromonadaceae. However, in no instance was a *V. cubensis* hindgut sample most closely related to its corresponding brown substance sample. *Laevicaulis alte* hindgut clustered with *V. cubensis* hindgut and the brown substance, with a high abundance of the same three Gammaproteobacteria (Figure 2.3). Verification of OTU identification with NCBI-BLASTN was inconclusive for lower classification of the Enterobacteriaceae, but suggests that the Aeromonadaceae may be *Aeromonas* spp. and confirms *Cronobacter dublinensis*. In the brown substance samples, approximately 5% of total reads were Aeromonadaceae, 22% were Enterobacteriaceae, and 12% were *Cronobacter dublinensis*. In *V. cubensis* hindgut samples approximately 7% of the read counts were Aeromonadaceae, 35% were Enterobacteriaceae, and 13 % were *Cronobacter dublinensis*. In *L. alte* hindgut samples approximately 14% of total reads were Aeromonadaceae, 22% were Enterobacteriaceae, and 15% were *Cronobacter dublinensis* (Figure 2.3).

The juveniles that hatched from eggs with brown substance do not all group together. One outlying juvenile sample is placed most closely to the soil samples. This sample contains a high abundance of *Prosthecobacter debontii* (21% of total reads), which is present in most other samples in very low abundance. Juveniles that hatched from eggs with brown substance most noticeably contain two types of Sphingobacteriia (*Sphingobacterium* and *Pedobacter*) and an

Alphaproteobacteria (Rhizobiales). These juveniles are distinct from the soil and contain the three primary Gammaproteobacteria found in adult hindguts and brown substance but in much lower proportions. Approximately 0.43% of total reads from juveniles hatched from eggs with brown substance were Aeromonadaceae, 0.1% were Enterobacteriaceae, and 0.07% were *Cronobacter dublinensis*. These three Gammaproteobacteria were found in most of the soil samples but at lower amounts than in the juveniles hatched from eggs with brown substance. Approximately 0.02% or fewer total reads from the soil samples represented these Gammaproteobacteria.

The diversity in the samples of juveniles from surface sterilized eggs was much lower than in all other sample types, including the juveniles from eggs with brown substance (Table 2.2). The heatmap (Figure 2.3) shows that these samples are distinct from the rest of the sample types. Juveniles from surface sterilized eggs contain high abundance of bacterial taxa that are found in very low abundance in all other sample types. Two of the juveniles from surface sterilized eggs are grouped with the juveniles hatched from eggs with brown substance and one sample is noticeably different from all other sample types. Juveniles from the same egg mass that was split in half to be surface sterilized or left with brown substance are not each others' closest samples. Additionally the juveniles from surface sterilized eggs did not have any Aeromonadaceae and only trace amounts of *Cronobacter dublinensis* (approximately 0.0001% of total reads), but did contain Enterobacteriaceae in proportions comparable to juveniles hatched from eggs with brown substance.

Table 2.1 Sampling depth of 16S from samples and Shannon diversity before and after rarefaction to 10,079. Low-quality samples were removed prior to analysis.

sample type	raw reads	Shannon Diversity Index prior to rarefaction	Shannon Diversity Index after rarefaction
V. cubensis hindgut 1	25493	1.93	1.92
V. cubensis hindgut 2	55675	2.78	2.76
V. cubensis hindgut 3	25499	3.17	3.15
V. cubensis hindgut 4	1010	-	-
V. cubensis hindgut 5	6280	-	-
brown substance 1	75629	3.51	3.51
brown substance 2	83936	2.92	2.91
brown substance 3	94948	3.56	3.55
brown substance 4	84463	3.62	3.57
brown substance 5	5435	-	-
L. alte hindgut 1	2	-	-
L. alte hindgut 2	80245	2.02	2.03
L. alte hindgut 3	11111	3.56	3.55
juveniles from eggs with brown substance 1	13001	2.71	2.71
juveniles from eggs with brown substance 2	61638	1.56	1.57
juveniles from eggs with brown substance 3	49648	3.53	3.49
juveniles from eggs with brown substance 4	11409	3.31	3.32
juveniles from eggs with brown substance 5	1704	-	-
juveniles from surface sterilized eggs 1	30268	2.06	2.07
juveniles from surface sterilized eggs 2	5516	-	-
juveniles from surface sterilized eggs 3	10079	2.50	2.50
juveniles from surface sterilized eggs 4	14016	2.73	2.74
juveniles from surface sterilized eggs 5	5234	-	-
soil (*L. alte*)	81436	4.47	4.45
soil 1	131595	4.48	4.44
soil 2	89685	4.08	4.07
soil 3	90372	4.09	4.05
soil 4	88237	4.61	4.59
soil 5	108064	4.41	4.40
soil 6	82297	4.39	4.39
control	9999	-	-

Table 2.2 Mean number of OTUs and range in each sample type. Outlying samples with low read counts were removed prior to descriptive analysis.

sample type	mean (range)
V. cubensis hindgut	189 (142-258)
brown substance	352 (299-398)
L. alte hindgut	190 (124-256)
juveniles from eggs with brown substance	141 (110-225)
juveniles from surface sterilized eggs	49 (39-57)
soil	468 (415-536)

Figure 2.2 Non-metric multidimensional scaling plot of sample types: brown substance, hindgut (both *V. cubensis* and *L. alte*), juveniles from eggs with brown substance, juveniles from surface sterilized eggs, and soil. Although overlapping, all clusters are significantly dissimilar from one another (ANOSIM (binary: R=0.6314, p≤0.001 and Hellinger: R=0.7459, p≤0.001) and adonis (binary p≤0.001 and Hellinger p≤0.001)).

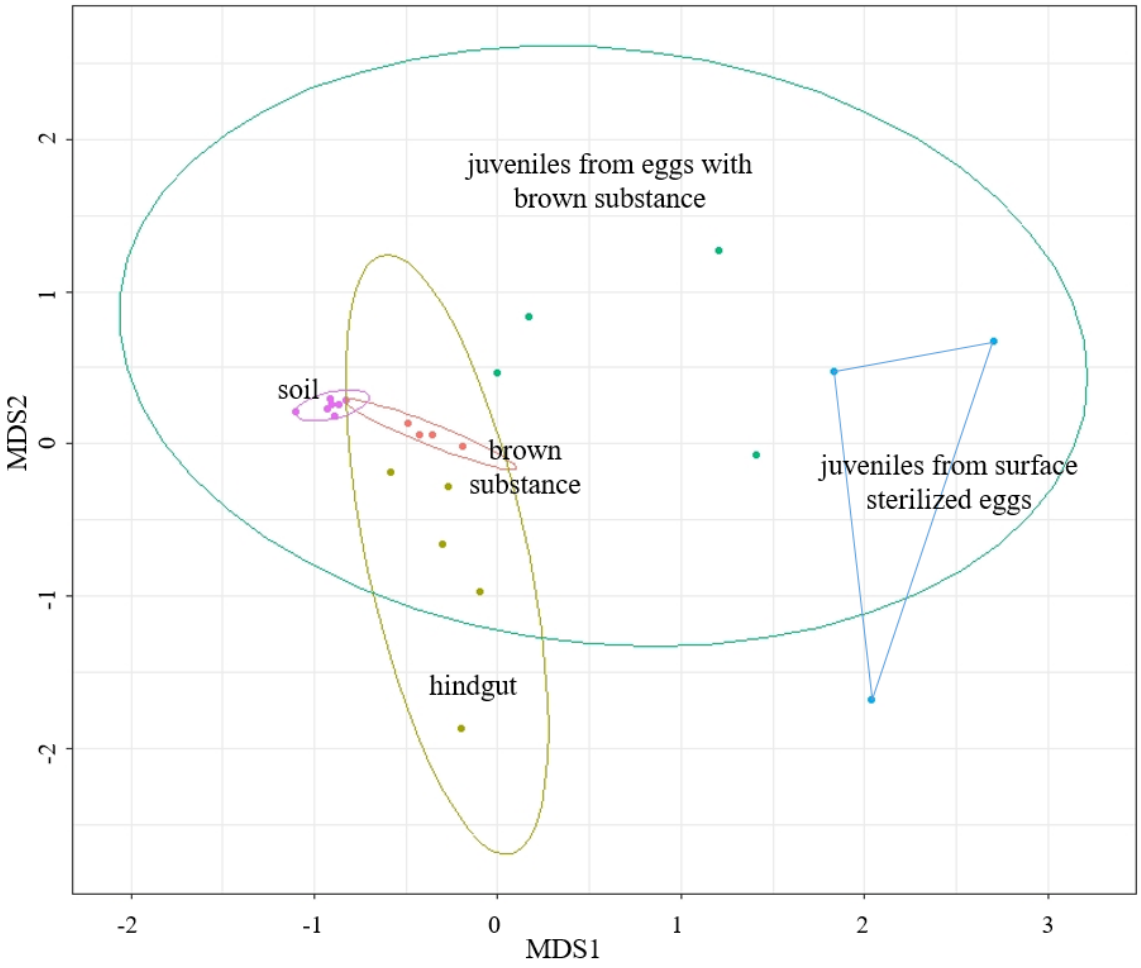
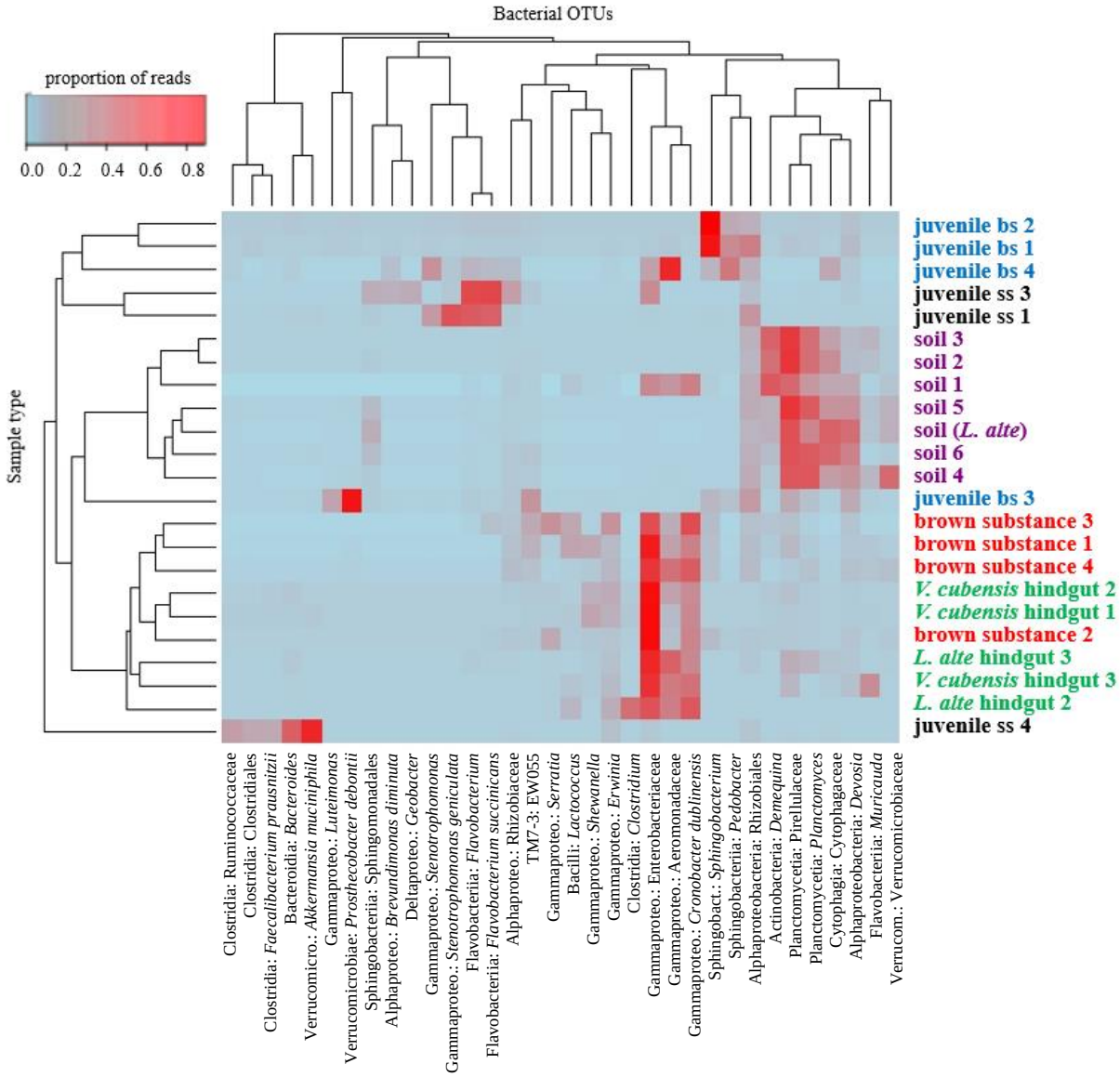


Figure 2.3 Heatmap showing abundance of bacterial taxa found in each sample. Data were rarefied to 10,079 reads. OTUs represented by fewer than 5% of read counts were excluded. Dendrograms are produced based on Bray-Curtis dissimilarity index. Sample type, represented as rows, are color coordinated. Juveniles hatched from eggs with browns substance are labeled as "juvenile bs" and juveniles from surface sterilized eggs are labeled as "juvenile ss". Bacterial OTUs, represented by the columns, may be abbreviated (e.g., Gammaproteobacteria = Gammaproteo.)



Discussion

This study provides the first characterization of *Veronicella cubensis* hindgut microbiota and the first analysis of the brown substance laid on egg masses by some Veronicellidae. It was predicted that similar microbes would be found in *V. cubensis* hindgut, brown substance, and juveniles hatched from eggs with brown substance, suggesting the use of an extracellular substance as a method of bacterial transmission between generations. Observations of lab reared slugs made during the course of this study showed that the brown substance is probably fecal material produced from ingested substrate. When housed on light brown damp paper towels, egg laying *V. cubensis* produced a brown substance that was light brown in color, corroborating this inference. In this study the brown substance was derived from soil ingested by the egg laying adult. As others have also concluded, the brown substance does not appear to be typical *V. cubensis* feces (Capinera & Guedes Rodrigues 2015). By passing through the digestive system the soil turned brown substance would have been inoculated with adult slug hindgut bacteria and therefore more closely clustered with hindgut samples than with soil samples in the heatmap (Figure 2.3). The brown substance and *V. cubensis* hindguts share high abundance of the same three Gammaproteobacteria taxa. Soil samples contain trace amounts of these three OTUs, with the exception of one sample (soil 1, Figure 2.3) that contains higher abundances. The most likely explanation for this outlying sample is that it contained slug feces. Because extracellularly transmitted bacteria can survive outside the host organism (Salem et al. 2015), the presence of these Gammaproteobacteria in the soil is expected.

Gammaproteobacteria exhibit a high diversity of forms and functions, from pathogens to insect endosymbionts (Williams et al. 2010). Two OTUs identified in the slug hindgut and brown substance are only identified to the family level, Enterobacteriaceae and Aeromonadaceae. Because the microbiome of veronicellids has not been previously investigated, the inability to be more specific than this suggests there may be undescribed species. These species might be symbionts and play a crucial role in the fitness of the slugs. *Aeromonas* spp., which may be the Aeromonadaceae taxa here identified, are occasionally found in association with aquatic invertebrates (Miñana-Galbis et al. 2004; Miñana-Galbis et al. 2007), but can also be pathogenic to invertebrates (Martin-Carnahan & Joseph 2005). The third OTU is *Cronobacter dublinensis*

from the family Enterobacteriaceae. The genus *Cronobacter* includes a number of species, all previously lumped together as *Enterobacter sakazakii* (Iverson et al. 2007; 2008), which are opportunistic pathogens in humans and particularly problematic when infecting infants (Bar-Oz et al. 2001). The role these bacteria may play in slug physiology was not assessed and requires further analysis, particularly to determine a lower classification of the two OTUs noted above.

Significant ANOSIM and adonis results demonstrate that the microbial communities from all sample types are distinct from one another. Even though the brown substance is soil that has passed through a slug's digestive system, it possesses a distinct bacterial composition. The brown substance has a high diversity of bacterial taxa similar to that of the soil (Table 2.2), suggesting that the brown substance may retain many of the soil's bacteria. We expected to see similarities between the brown substance and juveniles hatched from eggs with brown substance bacterial communities. However, the bacterial OTUs found in high abundance in both the hindgut and brown substance are found in the juveniles hatched from eggs with brown substance only in very low proportions, below 1% of total read counts, with the exception of one sample. Because juvenile were collected immediately after hatching, and juveniles do not consume the eggs nor brown substance, the sampled bacteria might represent an inoculation from contact when the juveniles were emerging from the eggs. The bacteria propagules may take time to grow and become established in the juvenile gut. To further understand the establishment of a core microbiome, future assays should sample a time progression of juveniles after hatching to determine a timeline of bacterial colonization.

By hatching juveniles from eggs either with brown substance or from surface sterilized eggs we could further assess the possibility of the brown substance acting as a vector for transmission. The bacteria found in the highest abundance in juveniles from surface sterilized egg samples were only found in very low abundance in all other samples (Figure 2.3). Juveniles that hatched from surface sterilized eggs had very low bacterial OTU diversity (Table 2.2). Washing with bleach removed or greatly reduced the Sphingobacteriia that are found on the juveniles from eggs with brown substance. However, Rhizobiales, a nitrogen fixer, remained present (Figures 2.3). Only two of the three Gammaproteobacteria from adult hindguts and brown substance are present in the juveniles from surface sterilized eggs. This suggests the surface sterilization

methods may not have been entirely effective. It may be possible that bacteria crossed the egg membrane in the week before sterilization. Although I cannot definitively state whether the brown substance is the major component of bacterial transmission I am able to conclude that the bacterial community found on the surface of eggs, which includes the brown substance, is a component of the bacterial communities that juvenile slugs acquire immediately after hatching.

Additional insight gained from this study was the similarity in hindgut microbiota between *V. cubensis* and *L. alte*. *Veronicella cubensis* is native to the Caribbean (Pfeiffer 1840) while *L. alte* is native to central Africa (Solem 1964). *Laevicaulis alte* does not produce a brown substance when laying eggs. Both species are common invasive slugs and seem to occupy a similar dietary niche, being generalist herbivores and detritivores. The ANOSIM and adonis results suggest that *V. cubensis* and *L. alte* hindgut samples are more similar to each other than they are to the other sample types. The similarity between the gut microbiomes of these species is particularly interesting in the context of these species acting as rat lungworm vectors. For reasons still unknown, *L. alte* tends to have greater *Angiostrongylus cantonensis* infection rates than *V. cubensis* does (Kim et al. 2014). Understanding the physiology and the role microbes play may provide useful information for understanding the host-parasite interactions that control infection susceptibility, with ramifications for human health. Literature on veronicellid behavior is unfortunately sparse, especially compared to that on other slug families, notably those that are pests in temperate regions (e.g. Barker 2002). A systematic review of all veroncellid species with analysis of the behavior of laying eggs with brown substance would provide information on its biogeographic and evolutionary origins. Sampling of other veronicellid slug hindguts may also shed light on the lower classification of the Enterobacteriaceae and Aeromonadaceae OTUs collected from hindguts and brown substance and may reveal a consistent symbiosis across the family Veronicellidae or within specific lineages only.

Conclusion

This study provides a first investigation of the microbiota of *Veronicella cubensis*, an extracellular substance possibly used for transmission, and a comparison with the confamilial species *Laevicaulis alte*. One primary question was the function that the brown substance placed on eggs by *V. cubensis* may have in inoculating juveniles with potentially beneficial microbial communities. We confirmed that the brown substance is substrate that is ingested and laid as fecal matter on the surface of the eggs. Three Gammaproteobacteria were consistently found in high abundance in adult *V. cubensis* and *L. alte* hindguts and brown substance. These bacteria were in low abundance in juveniles hatched from eggs with brown substance and in even lower abundance or not at all in juveniles hatched from surface sterilized eggs and the soil. Although there are strong similarities between hindgut samples and brown substance samples, they are distinct from one another (Figure 2.1 and 2.2). This similarity is probably a result of the origin of the brown substance as substrate. Most importantly, this study provides novel insights to guide further research into veronicellid microbiomes, which may be key to understanding the ecology, physiology, and parasitology of invasive slugs like *Veronicella cubensis* and *Laevicaulis alte*.

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